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THE RATE OF MULTIPLICATION OF *BACILLUS COLI* AT DIFFERENT TEMPERATURES.*

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IN estimating the rate of reproduction of bacteria, the following quantities must be known: the number of bacteria at the beginning of a definite period of growth, the number at the end, and the length of time over which the experiment extends. If the number at the start be represented by a, the number at the end by b, and the number of generations by n, we have the equation:

$$2^n = \frac{b}{a}.$$

Then if G be the time required to complete a full generation,

$$G = \frac{t}{n}$$

where t is the time covered by the experiment. The validity of the equation $2^n = \frac{b}{a}$ depends, of course, on the assumption that each bacillus divides into two nearly equal parts, and that all of the bacteria in a culture reproduce at nearly the same rate. In order that

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this even rate be kept up, conditions must remain constant during the experiment; and in order that the maximum rate for a given temperature be maintained, the following additional requirements, included in the list given by Buchner, Longard, and Riedlin, must be observed: A favorable liquid culture medium and a pure and actively growing culture must be used. The initial number of bacteria must be small, and the time of the experiment must not be long enough to allow an accumulation of products of metabolism sufficient to inhibit growth.

The estimation of the generation time of different species of bacteria has been attempted by various authors and by different methods. Nägeli and Schwendener[1] sought to use the amount of acid formed by certain fermenting bacteria as a criterion of numbers. Buchner, Longard, and Riedlin,[2] Rahn,[3] and Müller[4] used plate cultures for the determination of a and b in the above equation. Boland[5] used a turbidity standard in the estimation of numbers, and compared cultures with standard turbidity solutions made by talc suspensions. Klein[6] and Hehewerth[7] determined a and b by the direct counting of cultures stained in the moist condition.

My own work on this problem was attempted in the hope of obtaining more accurate results by the use of a new technique which I have developed in connection with other research.[8] Some modifications of this technique, developed in connection with the work here described, are as follows:

A large sterile cover is placed over the moist box (*b*, Fig. 1) and a drop of the culture to be used is placed under this cover. Drying is prevented by the saturated filter paper lining the sides of the moist box and by an abundance of water placed in the bottom. A moist atmosphere is further insured by placing numerous drops of sterile broth, gelatine, or agar on the under surface of the cover around the field of operation. The sterile pipette, *p*, the inner end of which is drawn out into a very fine capillary tip, is partly filled with the broth to be used and adjusted in the holder *kg*, in such a position that the tip may be brought into focus in the center of the field

[1] Das Microscop., 2. Aufl., Leipzig, 1877, p. 641.

[2] *Centralbl. f. Bakt.*, 1887, 2, p. 1.

[3] *Ibid.*, 1906, Abt. 2, 16, p. 417.

[4] *Archiv f. Hyg.*, 1903, 47, p. 127; *Ztschr. f. Hyg.*, 1895, 20, p. 245.

[5] Inaug. Dissert., Amsterdam, 1902.

[6] *Centralbl. f. Bakt.*, 1900, 27, p. 834.

[7] *Archiv f. Hyg.*, 1901, 39, p. 321.

[8] "On Heredity in Certain Micro-organisms," *Kansas University Sci. Bull.*, 1907, 4, No. 50, p. 3.

of the objective to be used. The culture drop containing the bacteria is brought into the field by means of the mechanical stage, and the pipette raised by screw *f* until its tip is in contact with the culture. One or more bacteria will enter the pipette by force of capillarity. The pipette is then quickly lowered, and, by moving the mechani-

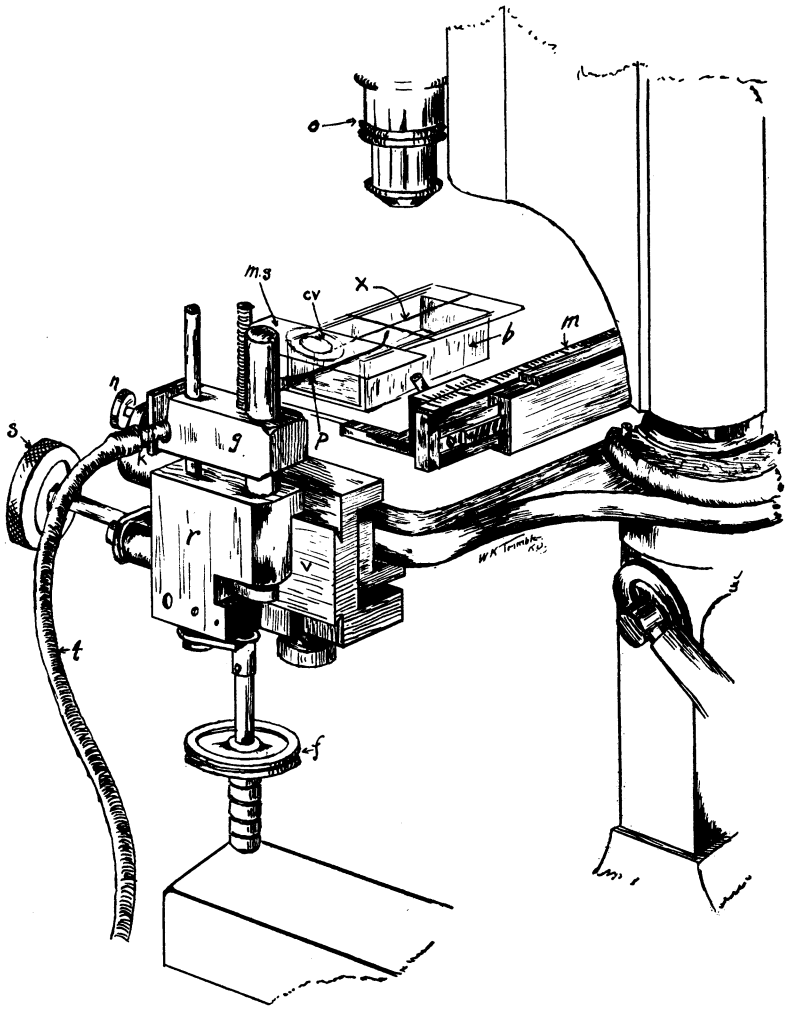

FIG. 1.

cal stage, a portion of the cover remote from the culture drop is brought into view. The pipette is then raised, and, by gently blowing into the rubber tube *t*, a portion of the contents containing the bacteria is discharged on the cover. If the bacteria are not too thick in the drop from which selection is made, and if the aperture of the pipette is sufficiently small, a single bacillus may be drawn into the pipette. If several are

taken up, it is easy to separate them by subsequent di utions on the cover. In preparing for a hanging drop series I have usually isolated several individuals, so that a selection can be made from the most actively growing culture at the second isolating. The further procedure is described below.

I have used in some of these experiments a modified pipette holder constructed to hold two pipettes. In this holder each pipette may be raised or lowered independently of the other, and they may be so adjusted that both capillary tips may be brought into the same field. An additional adjustment, provided with screws and arranged in block *g* (Fig. 1), allows these pipettes to be moved independently in and out, as well as up and down. This apparatus dispenses with the necessity of changing pipettes between the first and second isolations. However, in view of the ease with which a new pipette may be made and inserted, I have preferred for most experiments the simpler type of holder shown in the illustration.

When a bacillus is to be transferred to a test-tube, it is usually necessary to isolate the bacillus with one pipette and to transfer it to the test-tube with a fresh sterile one, so that one may be sure that there is but one organism transferred. A new pipette then must be made for each bacillus. In order to simplify this process and to use the same pipette for several transfers, the following modification of the process has been devised: A piece of mica, about 2×4 cm. in size, is cut out and in the center a round aperture about 8 mm. in diameter is made. Over this aperture a clean coverglass, about ½ in. in diameter, is placed and the cover, together with the mica holder, is sterilized in the Bunsen flame and placed over the moist box in the position shown in the illustration. (*ms* and *cv*, Fig. 1.) The mica slip, shown at the left side in the illustration, may be placed at the opposite end of the moist box. In either case the large cover, *x*, is slipped to one side or the other to accommodate it. A drop of sterile broth is placed under the smaller cover either after it is placed on the moist box or just before. Then by means of the pipette, already in position, a single bacillus is taken up from the droplet in which it had previously been isolated. The cover on the mica slip is then brought into the field by means of the mechanical stage, the pipette is raised, and the bacillus deposited in the drop of broth under the smaller cover. An ordinary platinum loop, somewhat flattened, is sterilized and dipped into sterile broth. The wetted loop is brought into contact with the top of the small cover, and the cover lifted and deposited, together with the hanging drop and single bacillus, in a tube of sterile broth. The mica may now be sterilized anew, again put into place with a new cover and a second bacillus isolated. The bacillus may be seen moving about in the droplet under the smaller cover, so that one is sure that it is in an active condition and is transferred in this state to the test-tube.

This method may be used either with or without the warm box described below. A bacillus may be isolated, and left over the moist box protected by the warm box until two generations have been formed. Then each of the four bacilli may be taken out and deposited in four separate test-tubes, using the same pipette for each. Contamination may be avoided by arranging a hood of moist cloth or paper over the portion of the microscope used in the process. In the course of many isolations by this method, I have thus far had no contaminations. The method is a convenient one for isolating single organisms from mixed cultures. The single organism may be transferred to liquid or solid media.

The warm box used is made on the plan of the Zeiss warm box with metal base, glass front, and hinged, wooden sides. Heat, in my apparatus, is maintained by

resistance coils placed under the base of the microscope, and the temperature is kept constant by an electro-thermoregulator arranged to automatically connect or disconnect the heating current. I have protected the top and sides of the box with a jacket made of two layers of felt with asbestos wool between the layers. In this apparatus a very constant temperature may be maintained.

In preparing for an experiment the microscope is placed in the box, the light properly focused, and the pipette adjusted in position under the cover-glass holding the hanging drops. Of the microscope only the tube and the fine and coarse adjustments project from the closed box. Apertures are provided for the rubber tube, *t*, and for rods by which the mechanical stage is manipulated. A small door is placed in the left side of the box in order that the thumb may be inserted for manipulating screw *f* of the pipette holder. So a series, involving many isolations of bacteria, may be continued for hours without opening the box or altering the temperature.

There is a tendency, in the warm box, for moisture to collect on the part of the large cover which lies immediately over the center of the stage, since the heat, coming from below, passes around the condenser and causes the moisture in the bottom of the glass box to form a slight vapor. So, by moving the portion of the large cover holding the series of hanging drops to the right or left of the center of the stage immediately after an observation, the amount of moisture on that part of the cover may be nicely governed.

In order to carry on this work successfully, it is necessary to work with a strong light, especially when the warm box is used. For this I employ a Welsbach burner; and, in order to partially free this light from yellow rays and to focus it strongly on the mirror, I use a large spherical glass globe condenser filled with a weak solution of copper sulphate.

Much depends on using pipettes with a very small aperture at the capillary end. I have somewhat improved the method of making these pipettes and proceed as follows: One end of a thin-walled glass tube, 8 to 10 cm. in length and about 4 mm. in diameter, is drawn out in a small flame into a thin-walled capillary tube about 0.5 mm. in diameter. The capillary end is again drawn out over a very small, narrow flame produced by a micro-burner. This small flame should be only 3 or 4 mm. in height. If the capillary tube is held at a proper distance above this flame and drawn out with some force the instant the glass softens, apertures of very narrow diameter and with smooth edges may be made. About 5 mm. of the tip is then bent at right angles in the flame and the pipette is ready to be filled with the medium. This may be taken directly from a test-tube. If, on inserting the tip into the liquid, it is found that there is no aperture present, one of sufficient fineness may often be made by scratching the tip *very gently* on the sides of the test-tube. The size of the aperture may be gauged approximately by the rapidity with which the liquid enters the pipette when suction is applied through the rubber tube. One may make and successfully use pipettes having an aperture not exceeding a small yeast cell in diameter. Much coarser ones may be successfully used, but where the aperture is too large the labor is much increased and the accuracy of the work diminished.

The essential feature of the method I have employed is that each experiment is begun with a single bacterium. Thus a of the equation given above is reduced to unity, the equation simplified to $2^n = b$,

and the errors incident on the determination of a are reduced to the minimum. Further, on account of the small magnitude of a, a considerable time may be employed and a considerable number of generations formed while b is still relatively a small number. This facilitates the determination of b; and, in making n relatively large, reduces the margin of error. Moreover, the relatively small number of bacteria in the culture minimizes the danger of inhibition of growth due to the accumulation of products of metabolism. Again, when a considerable number of bacteria are used as a starter, there are likely to be some less vigorous ones included, however active the culture from which the starter is taken. By selecting as a starter a single healthy bacterium from the most vigorous individuals of a culture, one may avoid this error and begin the experiment under optimum conditions. The term "single bacterium" is made to include some cases in which two or more elements were joined in a short chain.

One race of *B. coli* was used during the whole work, a strain obtained from the collections of the Pasteur Institute in 1904. During the two years or more during which these experiments were conducted the growth rate of this strain of *B. coli* was frequently tested under similar conditions of medium and temperature, and there was no evidence of degeneration or other change. In practically all experiments, beef peptone broth was used.

Three variations in the above method were used in ascertaining the growth rate. The bacilli were cultivated in hanging drops over hollow slides, in a series of hanging drops kept in a warm box, and in test-tubes. In the warm-box and test-tube series only a limited number of temperatures was employed, since these methods were used largely to confirm the results obtained by the hanging-drop method.

In the hanging-drop method the following procedure was followed in most cases. A single bacillus was isolated and allowed to stand in the medium and at approximately the temperature at which it was subsequently to be grown, until one, two, or sometimes three generations had formed. Then with a fresh pipette, the two, four, or eight similar units were distributed each into a separate hanging drop, and one of these units killed and stained to serve as a control of the size of the others. The cover was then sealed over a hollow

slide, placed at the appropriate temperature, and left a definite period of time. Temperatures above room temperatures were, in a majority of cases, maintained in an incubator or water bath heated by electric heat governed by an electrothermostat; so that these temperatures were in most cases very constant. Below room temperature, various devices, usually depending on cold water, either flowing from the tap or otherwise arranged, were employed. In a few cases, a refrigerator was used, but this was not found to be sufficiently reliable.

At the close of an experiment the cover-glass was again placed over the moist box and the cultures were killed and stained by adding to each hanging drop a small quantity of a reagent composed of a saturated solution of methylene blue to which was added, just before use, a small quantity of a 5 per cent solution of potassium hydrate. The addition of this reagent was made under the microscope and with the aid of the pipette previously used in isolating, or of a similar pipette, so that a small enough quantity could be used to instantly kill and stain without adding enough color to the drop to interefere with the counting. The counting was done in the hanging drop and the comparison made with the control in order to estimate generations in units of the same size as those used in the starters. If the generations did not come out even in a given series, the time was usually varied in the next experiment so as to make the number of generations come out approximately even. In some instances no control was kept, and the size of the starter estimated by direct measurement with the eyepiece micrometer. Tests were usually confirmed by experiments made at the same time and temperature and with the same medium. In many cases, further confirmatory tests were made at the same or approximate temperatures and with a different lot of medium.

The results of these experiments are given in Table 1. In some cases the values in the n-column are not whole numbers. Here the decimal was reckoned on the basis of the size of the units at the end of the series as compared with the control. In other cases two even values of n are given for the same experiment, values between which the true value lies. Such numbers are inclosed in brackets in which are also given the appropriate values of G.

The numbers in the fifth column indicate the number of hanging drops which gave the values which precede. If the number 2 is given it is meant that the corresponding experiment was done in duplicate; if 3, in triplicate, and so on. The numbers marked with asterisk in the *G*-column are those considered to be the minimum reliable values, and it is largely on the basis of these marked numbers that the curve given below is plotted. These values are selected because determined by experiments conducted under optimum conditions and apparently relatively free from error. Further, these selected values were in most cases confirmed by a considerable number of duplicate experiments. Since the intention of the experiments is to obtain the maximum rate rather than the average rate, these selected values are probably more reliable than averages, though the averages at most of the temperatures closely approximate the values selected.

Practically all the determinations made during the work are given in the table, the ones which vary greatly from the minimum as well as the ones which closely approximate it, since it is desired to show the amount of variation in the results. Only certain determinations made while the method was being developed, or results based on experiments known to be faulty, are omitted.

It is shown in Table 1 that the growth of this race of *B. coli* begins at about 10° and the rate rises rapidly to about 37°5 where it reaches a minimum generation time of nearly 17 minutes. It remains at a nearly constant high rate until about 45° when it falls rapidly and nearly ceases at 48°. The temperature curve is shown much more graphically in the chart given below and further discussion of it is deferred to that part of the paper.

The values obtained for *G* in the lower temperatures vary greatly. This is due in part to the degeneration often noted in the cells at very low temperatures. It is also partly due to the difficulties of keeping constant low temperatures for the long time necessary to obtain a considerable number of generations. For when only two or three generations occur in a relatively long space of time, the margin of error is great. The irregularities observed in the rate of reproduction in the higher temperatures were correlated with degeneration of cells, tendencies to grow into long threads, and

TABLE 1.

MULTIPLICATION OF *B. Coli.* HANGING-DROP METHOD.

Temperature	Time in Minutes (*t*)	No. of Gen. (*n*)	Generation Time in Minutes (*G*)	No. of Deter.	Temperature	Time in Minutes (*t*)	No. of Gen. (*n*)	Generation Time in Minutes (*G*)	No. of Deter.
5.5–6.2		Growth			30.0	208	7	29.7*	4
5.8–6.4	7 hrs.	none or			30.2	180	6	30	2
5.9–6.4	5¼ hrs.	very			30.5	165	6	27.5	2
7.0–8.5	5 days	slight					8	30.0	
9.0–11.0	25 hrs.	2	750	4	31.6	240	9	26.7	1
9.5–10.5	1044	2	522	2	31.6	253	8	31.6	1
10.0	2595	3	865*	2			17	24.7	
12.5	1140	2	570*	2	31.6	420	16	26.3	1
12.6–13.9	360	2.2	164		31.6	202	8	25.3	1
13.6–14.3	755	3.5	214*	2	31.6	196	8	24.5	1
13.3–13.8	586	3	195	1	31.6	175	7	25.1*	4
		3.5	182		32.0	150	6	25.0	5
14.2	1285	6	214	1	33.6	257	10	25.7	4
		6.5	198*				11	23.4	
14.3	1168	6	194	4	33.6	250	10	25.0	1
		6.5	180		33.6	185	8	23.1	1
15.4–15.8	805	5	161	5	33.6	125	5.5	23.7	4
16.0–16.8	1570	13	120.8*	2			5	25	
18.1	470	5	94	6	33.6	128	5.5	23.3	6
18.5–18.8	565	5.2	108	2			5.0	25.0	
18.5	490	5	98*	4	33.6	115	5	23.0*	3
19.5	390	4	97.5	1	33.6	132	5	26.4	2
19.5	558	5.3	105.3	4			6	22.0	
19.5	765	8	95.6*	1	33.6	120	5	24	2
21.5–21.8	355	5.7	62.2	3	33.6	71	3	23.7	3
21.6	420	7	60*	5	33.6	77	3	25.6	3
22.6–23.0	318	6	53*	3	33.6	240	11	21.8	2
23.6	226	5	45.2	2	33.6	141	6	23.5	2
23.6	407	8	50.9*	4	33.6	132	6	22.0	1
23.6	454	9	50.4	2	33.6	226	10	22.6	2
23.6	348	7	49.7	1	34.0	120	5.3	22.6	2
23.6	405	8	50.8	1			5.5	21.8	
23.6	360	7	51.4	1	34.4–35.6	160	7	22.9	1
23.7–24.1	276	5	54.6	3	35.0	145	6.5	22.3	1
24.1	240	4.8	50*	1	35.0	170	7	24.3	1
24.6–25.5	164	4	41*	4	35.0	136	6	22.6	2
25.2–26.0	205	5	41	2	35.0	120	5.5	22.0	2
25.5	362	9	40.2	1	35.0	132	6	22.0*	2
25.5	443	11	40.3*	2	35.0	187	8	23.4	1
25.5	303	7	43.3	1	35.0	187	7	25.5	1
		8	37.9		35.0	187	8.5	22.0	1
25.5	333	8	41.6	1	35.0	145	6.3	23.0	1
25.5	423	19	42.3	1	35.0	120	5	24.0	1
25.5	410	10	41.1	2	35.0	117	4.8	24.0	1
27.6	183	5	36.5	1	35.0	134	6	22.3	1
27.6	238	6	39.7	1	35.0	130	6	21.8	1
27.6	212	6	35.3	1	35.1–35.2	148	6.5	22.8	1
27.6	230	6	38.3	1	35.1	132	6	22	4
		7	32.9				6.5	20.3	
27.6	395	11	35.9*	1	35.1	120	5	24	4
27.6	494	14	35.3	1			5.5	21.8	
27.6	404	11	36.7	1	36.0	120	6	20.0*	4
		12	33.7		37.0	120	6	20.0	6
27.6	251	7	35.9	1	37.0–37.4	120	6.2	19.4*	2
27.9–28.9	185	6	30.8	4	37.2–37.4	120	6.5	18.5	4
29.5	180	6.5	27.7	3	37.5	111	5.5	20.2	1
		6	30		37.5	120	7	17.2*	7
29.6	156	5	31.2*	1	37.5	185	9	20.6	1
29.6	163	5	32.6	4	37.7	131	7	18.7	2
		13	28.9		37.7	121	6	20.2	5
29.6	376	13.5	27.9	1	37.7	233	11	21.2	1
		14	26.1		37.7	116	6	19.3	2
29.6	180	6	30	3	37.7	106	5	21.2	2
29.6	162	5	32.4	3	37.7	155	8	19.4	2
29.6	311	11	28.3	3	37.7	101	5	20.2	1
29.6	493	15	38.2	2	37.7	100	5	20.0	8
29.6	420	13	32.2	1	37.7	99	5	19.8	2
29.6	311	10	31.1	2	37.7	120	6	20.0	6

TABLE 1.—*Continued.*

Temperature	Time in Minutes (t)	No. of Gen. (n)	Generation Time in Minutes (G)	No. of Deter.	Temperature	Time in Minutes (t)	No. of Gen. (n)	Generation Time in Minutes (G)	No. of Deter.
37.7	98	5	19.6	1	44.5	150	6	25	3
38.1–39.2	127	6	21.2	1	44.5	138	7	19.7*	3
38.2	120	6	20	1	44.6	146	7	20.9	3
38.5	120	7	17.2*	11	44.6	169	7	24.1	1
38.5	120	6	20.0	5	44.6	122	6	20.3	4
38.5	117	6	19.5	2	44.6–44.8	110	5	22.0	5
38.5	119	6	19.8	3	44.6–44.8	125	6	20.8	5
38.5	132	7	18.4	1	44.8	125	6	20.8	1
		8	17.4				7	17.9	
38.5	139	7	18.9	1	44.8	220	12	18.3	1
38.5	119	7	17.0	1	44.8	220	11	20.0*	2
38.5	174	9	19.3	1	44.8	159	7	22.7	2
38.5	154	9	17.1	1	44.8	127	6	21.2	1
38.5	172	9	19.1	1	44.8	127	7	18.1	1
38.5	171	10	17.1	1	44.8	136	6	22.7	2
38.5	102	5	20.4	1	44.8	102	5	20.4	1
38.5	130	7	18.6	2	45.0	129	6	21.5	1
38.5	125	7	17.9	1	45.2	99	4	24.8	
38.5	183	10	18.3	1	45.2	120	4	30.0	1
38.5	123	7	17.6	3	45.2	120	5	24.0	1
38.5	158	9	17.6	1	45.2	120	6	20.0*	3
39.6	119	7	17.0	1	45.2	268	9	29.8	1
39.6	121	7	17.3	2	45.2	91	4	22.8	2
39.8–40.2	120	7	17.2	3	45.2	120	4	30	1
40.7	180	5	36.0	1	45.6	137	6	22.8	3
		6	30.0		45.6	114	4	28.5	3
40.5	120	7	17.2*	5	45.8	102	5	25.0	1
40.8	150	6	28.3	4	45.8	102	3	34.0	2
		7	24.3		45.9–46.1	93	3	31.0	1
40.8	120	7	17.2	1	45.9–46.1	171	7	24.4	3
40.8	190	10	19.0	1	45.9–46.1	171	6	28.5	2
40.8	124	7	17.7	3	46.0–46.2	120	2	60.0	1
40.8	120	7	17.2	4	46.3–46.5	142	5	28.4	3
42.8	118	6	19.7	2	46.5	165	6	27.5	1
42.8	120	6	20.0	3	46.5	152	3	50.6	2
42.8	156	8	19.5	1	46.8	137	2	68.5	1
42.8	156	7	22.3	2	46.8	137	3	45.6	1
43.3–43.8	120	6.8	17.7*	2	46.8	183	4	45.7	1
44.2	128	6	21.3	2	46.8	180	4	45.0	1
		7	18.3		46.8	457	10	45.7	3
44.2	116	5	23.5	1	46.8	457	11	41.5	2
44.2	122	5.5	22.1	4	46.9–47.3	195	1	2 genera	tions
		6.0	20.3		47.0–48.5	Growth slight and irregular			
44.3	124	6	20.7	3	50.0	No growth observed			
44.5	125	5	25.0	2					

other morphological irregularities. These with the failure of some of the cells to grow make determinations in the region above 45° comparatively unreliable.

In the second method the warm box described on p. 382 was used. Here the cover-glass holding the isolated bacilli was not removed from the moist box (see *b*, Fig. 1) on the stage of the microscope, and the experiment was kept under observation during the whole time of a series, sometimes extending over a space of 12 hours. A single bacillus, well accustomed to the temperature and medium and vigorously growing, was isolated and allowed to grow to usually two and sometimes three generations. Then one of the daughter

cells was removed by the pipette and placed alone in a new drop. When this had attained two or three generations one of its descendants was isolated, and so on. Thus, long series were carried out, the whole series kept in the warm box under nearly constant temperature and conditions of moisture, and exposed to the light only during the brief time required to make the isolations—often less than two minutes. At each fresh isolation, the time, temperature, approximate size of the unit transferred, and its motility were noted; so at the close of a long series not only the value given by dividing the whole time by the whole number of generations could be obtained, but values for lesser periods when the rate of reproduction may have varied. In a majority of the series the rate was fairly constant

TABLE 2.

MULTIPLICATION OF *B. Coli.* WARM-BOX METHOD.

Temperature	Time Whole Series (t)	Number of Generations Whole Series (n)	Generation Time Whole Series (G)	Time Partial Series (t_1)	Number of Generations Partial Series (n_1)	Generation Time Partial Series (G_1)
29.5–30.8.....	320	9	35.6	184	6	30.7
				123	4	30.8
29.8–30.4.....	335	11	30.5			
30.0–30.4.....	366	11	33.3			
33.0–33.8.....	736	30	24.5	223	10	22.3
				455	19	23.9
33.6–34.2.....	360	16	22.5			
35.8–37.4.....	525	26	20.2			
36.2–37.0.....	389	20	19.5	303	16	18.9
				236	12	19.6
36.6.....	205	9	22.8	148	7	21.1
37.1.....	345	15	23.0	152	7	21.8
					8	19.9
37.1–38.2.....	491	22	22.3			
37.1–39.6.....	543	30	18.1	201	11	18.3
				342	19	18.0
				141	8	17.6
				327	18	18.2
37.4–38.4.....	212	11	19.3	140	8	17.5
37.4–38.1.....	313	17	18.4	219	12	18.3
				179	10	17.9
				132	7	18.9
37.6–38.6.....	427	20	21.4	427	20	21.4
				125	6	20.8
37.6–38.4.....	303	16	19.0	240	13	18.5
37.6–37.9.....	414	20	20.7			
37.9–38.1.....	396	18	22.0	146	7	20.9
				118	6	19.6
				214	12	17.8
				131	8	16.4
38.1–39.1.....	254	15	16.9	172	10	17.2
				82	5	16.4
				213	13	16.4
38.1–38.3.....	240	12	20.0			
38.3.....	720	38	18.9			
38.3.....	201	10	20.1			
39.3–41.4.....	750	38	19.7	550	29	19.0
				459	24	19.1
40.3–40.9.....	486	24	20.3	126	7	18.0
43.8.....	240	10	24.0	182	8	22.5

throughout the whole time; but in some, on account of temporary lack of moisture or some other unfavorable condition, the rate sometimes fell temporarily below the maximum. In these series I have given in Table 2 not only the value for the whole series, but also values for partial series during which conditions were more favorable. I have also (for the sake of confirming the results of the whole series) included some partial series taken from experiments in which the rate was nearly constant. In all series, whether whole or partial, it was necessary, in fixing the end points, to take bacilli of approximately the same size.

It is seen on comparing results that the values obtained by this method confirm those of the hanging-drop series. The uniformity of rate of division in the longer series is worthy of note. Up to the thirty-eighth generation at least, there is no period of rest or slackening of the growth rate, and it is probable that this rate would continue indefinitely so long as conditions were kept constantly favorable.

In order to eliminate possible error due to varying concentration of the medium in the hanging drops, the isolated bacterium was, in some series, drawn into the pipette and allowed to divide there into two or four. All were then discharged on the cover and one alone of the number drawn into the pipette for further growth, and so on. These series gave approximately the same results as when division was allowed to proceed on the cover-glass.

In the two methods described above, growth took place in hanging drops. In a third method, the single bacillus, vigorously dividing and accustomed to the medium, was transferred directly to a test-tube containing usually 5 or 10 c.c. of sterile broth. This tube was then incubated at the desired temperature until the first cloudiness appeared, which was usually after eight to nine hours at the optimum temperature. The culture was then killed by the addition of two or three drops of formalin, and the bacteria counted unstained in the ordinary Zeiss or Leitz counting chamber. The size of a bacillus used as a starter was noted and this size taken into account in the final estimation of generations.

The number of bacteria, *b*, given in the third column (Table 3) includes the total number formed in the test-tube, and varies with the quantity of broth as well as with the cloudiness of the culture. In the

fourth column under n_1 and n_2 are given the even powers of 2 lying above and below the corresponding b, and under G_1 and G_2 are given the generation times corresponding to n_1 and n_2. But one of these values is given where b closely approximates an even power of 2. In the sixth and seventh columns are found the values of n and G worked out according to the formulas given at the top of the respective columns.

TABLE 3.

MULTIPLICATION OF *B. coli* IN TEST-TUBE CULTURE.

Temperature	Time in Minutes (t)	Number of Bacteria Millions (b)	Number of Generations (n_1)	(n_2)	Generation Time in Minutes (G_1)	(G_2)	$2^n=b$ (n)	$G=t/n$ (G)
30.0.....	775	94.2	27	26	28.7	29.8	26.48	29.3
	762	81.8	27	26	28.2	29.3	26.28	29.0
	770	70.0	27	26	28.9	29.6	26.06	29.5
	820	60.0	26	25	31.5	32.8	25.84	31.7
	835	57.8	26	25	32.1	33.4	25.78	32.4
35.0.....	573	126.5	27	26	21.2	22.0	26.71	21.3
37.0.....	609	1240.0	31	30	19.7	20.3	30.28	20.1
	538	111.75	27	26	19.9	20.7	26.74	20.1
36.5–37.5.....	522	98.41	27	26	19.3	20.1	26.55	19.7
	454	15.62	24		18.3		23.90	19.0
	465	30.40	25		18.6		24.86	18.6
	504	102.88	27	26	18.7	19.4	26.28	19.2
	484	38.46		25		19.4	25.19	19.2
37.0–37.3.....	532	144.7	28	27	19.0	19.7	27.18	19.6
	546	164.5	28	27	19.5	20.2	27.29	20.1
	511	103.5	27	26	18.9	19.7	26.62	19.2
	506	128.0	27	26	18.7	19.5	26.93	18.8
37.2–37.5.....	561	169.67	28	27	20.0	20.8	27.34	20.5
	478	135.0	27		17.7		27.09	17.7
	515	102.14	27	26	19.1	19.8	26.61	19.4
	534	324.9	29	28	18.4	19.1	28.28	18.9
37.2.....	514	79.14	27	26	19.0	19.8	26.24	19.6
	483	30.12	25		19.3		23.84	19.4
	491	51.45	26	25	18.9	19.7	25.63	19.2
37.3.....	535	60.46	26		20.6		25.52	21.0
37.5.....	569	156.0	28	27	20.3	21.1	27.22	20.0
	549	333.0	29	28	18.9	19.8	28.31	19.2
	507	108.0	27	26	18.8	19.5	26.69	18.3
	535	69.5	27	26	19.8	20.6	26.05	20.5
	549	102.0	27	26	20.3	21.1	26.61	20.6
	538	97.0	27	26	19.9	20.7	26.53	20.3
	553	66.0	26		21.3		25.97	21.3

It is seen that the results obtained in the test-tube cultures closely approximate those obtained by the other methods. It is evident by comparing G_1 and G_2 that the probable error in estimating numbers is small; for an error of 100 per cent in counting will amount to an error of only one generation, about 5 per cent in the value of G, and the average error in counting is probably under 10 per cent.

A certain proportion of the test-tube cultures inoculated with single bacilli failed to grow. This is more probably due to some error in transferring than to any effect of a large quantity of sterile

medium on a single bacillus. In the last series conducted with the most improved technic, all of a series of six bacilli grew when transferred each to a tube containing 5 to 10 c.c. of broth. A number of earlier failures were probably due to allowing the media surrounding the isolated bacillus to concentrate by partial drying before the bacillus was transferred. The newly formed bacilli seem especially sensitive to drying or excessive concentration of the medium.

Each of the three methods tends to eliminate the sources of error of the other two. The chief source of error of the first method, the smallness of the number of generations counted, is avoided in the second and third methods where the generation number is large. Inhibition of the rate of growth due to crowding is avoided in a warm-box method where usually no more than four bacilli are found in a drop at one time; and the agreement of the results obtained by this method with those of the other two methods makes it seem unlikely that either in the hanging drops or test-tubes a sufficient number of bacteria were allowed to form to inhibit growth by crowding. The estimation of the number of bacteria at the close of the experiment, while approximately exact in the test-tube experiments, can be made very exact in the hanging drops; and error from this source is avoided altogether in the warm-box method.

In the warm-box experiments where the one bacillus that is to serve as a starter for the next drop is chosen from two, four, or eight, there is a possibility of error in unconscious selection of larger or smaller elements, since division does not always result in the formation of exactly equal parts. This error has been avoided largely by selection of units of average size. This has been facilitated by isolating in some cases more than one of the units of a given drop and comparing the results of growth two or three generations later, thus following out the series in duplicate or triplicate. However, to ascertain the amount of error possibly due to unconscious selection, two series were carried out at the same time and under the same conditions, both series starting from a common ancestor. In one of these the largest of the units was selected at each transfer, in the other, the smallest. At the end of the entire time of the experiment, 476 minutes, the series in which the larger elements were selected gave 26 generations with a generation time of 18.3 minutes.

The series in which the smaller elements were chosen gave 23 generations, with a generation time of 20.7 minutes. So from this experiment in which the difference is less than 2.5 minutes, it may be concluded that the average error is not large when pains are taken to avoid selection.

Rahn, Hehewerth, and Müller[1] mention a phenomenon said to occur in their experiments which, if present here, would be an additional source of error. This is an inhibition, or even temporary stoppage, of growth occurring during the time immediately following the inoculation into the fresh broth of the bacteria used as a starter. The growth rate, at first very slow, is said to reach a maximum only after some time, and this maximum rate can continue only a comparatively short time until growth is checked by the accumulation of products of metabolism. This initial slow growth is the more marked the lower the temperature, and is less conspicuous when fresher cultures are used as starters and a larger number of bacteria sown. Rahn explains the more rapid growth following the initial pause by the production of a specific substance, comparable with the "bios" of Wildier, a substance that is produced by the bacteria and is necessary for their most vigorous growth.

In my experiments with *B. coli* I found absolutely no sign of this initial slow growth when rapidly dividing elements, accustomed to the medium, were used at the start. Several experiments were carried out in the warm box which clearly showed the absence of the initial slow growth. In these series the daughter cells were transferred to a new drop of fresh medium at the completion of the first division. Each hanging drop is then one generation older than the preceding, and the drops are made large enough to accommodate six or seven generations without crowding. After about two hours at the optimum temperature all the bacteria in the hanging drops of a series thus made are killed and stained. The resulting numbers in a series are shown in the following diagram:

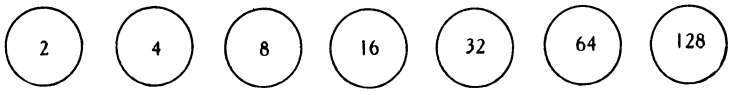

FIG. 2.

[1] *Loc. cit.*

If two generations are allowed to form in any one drop and three instead of one left behind at transferring, the three continued to divide at the same rate as the others but according to the series: 3, 6, 12, 24, 48, 96, etc. If there is an increased rate of reproduction after a considerable number of bacteria are formed we would expect a larger number than 64 or 128 in the older drops. In large hanging drops this series may be continued one or two generations further without evidence of any increasing rate of reproduction.

Further, if a certain concentration of bacteria is necessary to rapid growth, one would expect a very slow growth for some hours following the introduction of a single bacillus into 10 c.c. of broth. That this is probably not the case is shown by the fact that the generation time of such cultures for a long series is approximately the same as that in hanging drops where a considerable concentration of bacteria is soon reached. Further, in the warm-box experiments the rate is as rapid as in the hanging drop or test-tube, though in this case fresh broth is added with each transfer and the transfer may be made before more than two bacteria are formed in a drop. These experiments certainly argue against the theory that some specific substance must be formed in cultures of *B. coli* before reproduction can reach a maximum. It could hardly be maintained that enough of this substance is carried over with a single bacillus inoculated into 10 c.c. of broth to insure rapid growth, when it is remembered that the bacillus is isolated in fresh broth immediately before transferring. If quantities so minute as this are sufficient, no inhibition of growth would be expected when, as in experiments by the older methods, many hundreds of bacteria may be used in the starter.

A possible explanation of the initial slow growth mentioned in some published results is brought out by the following experiment:

Isolations were made of single bacilli taken from a hanging drop in which the bacteria, still actively motile, had assumed the small, short form characteristic of the *B. coli* in older cultures. On the same cover-glass and under the same conditions larger units, taken from young actively dividing cultures, were isolated. Each of these two kind of units, if plated, would give a single colony and be counted as one. At the end of 75 to 80 minutes the hanging-drop cultures formed from the different types of bacilli were stained and counted.

The single bacilli from the older hanging drops had formed, in two cases noted, only one large unit each, and in no drop more than five pieces, while the bacilli from the younger hanging drop had formed from 14 to 20 pieces, each capable of forming a colony in gelatine or agar. So a series in which the beginning and end numbers are determined by plate cultures would show an apparent initial period of slow growth if there were any bacilli of the small type in the starter. Even if the starter were taken from a very young culture there would likely be a proportion of these small forms present which would require, possibly, the time of several generation periods before they grew to a size sufficient for separation in the new medium. Experiments in which the numbers in the starter are estimated by direct observation of stained bacteria instead of by plate cultures would also show the apparent slow initial growth, due to the cause mentioned above. This apparent delay in growth might be greater in these experiments, for, since a considerable number of bacteria must be formed in a culture before they may be estimated by staining, older cultures must be used as starters. The initial delay then, according to my experiments, is due not to a cessation of growth, but to the time required by the shorter elements characteristic of older cultures to assume a size sufficient for division into two new units. I use the term ""division" for separation. It is probable that several partition walls may be formed before separation takes place.

I do not say that this explanation alone can account for the initial slow growth described by various authors, for I have not repeated all their experiments with the same organisms they use and under the same conditions. I can only say that in my experiments with *B. coli* there was no evidence whatever of this initial slow growth, when actively dividing bacteria were selected at the start. Some experiments conducted with *B. typhosus* and *B. subtilis* in the warm box (see below) indicates clearly that an initial period of slow growth is lacking in these species also.

Summing up all possible sources of error, it may be considered conservative to estimate the probable error of the determinations approaching the optimum temperature, that is, between 35° and 45°, as little exceeding 5 per cent or one minute in the generation time.

For temperatures much above or below these limits the error is much greater. In this estimate I refer to the most reliable minimum value, the ones marked with the asterisk in Table 1.

The curve plotted below is a recapitulation of the results obtained by all methods. The values are taken largely from the numbers

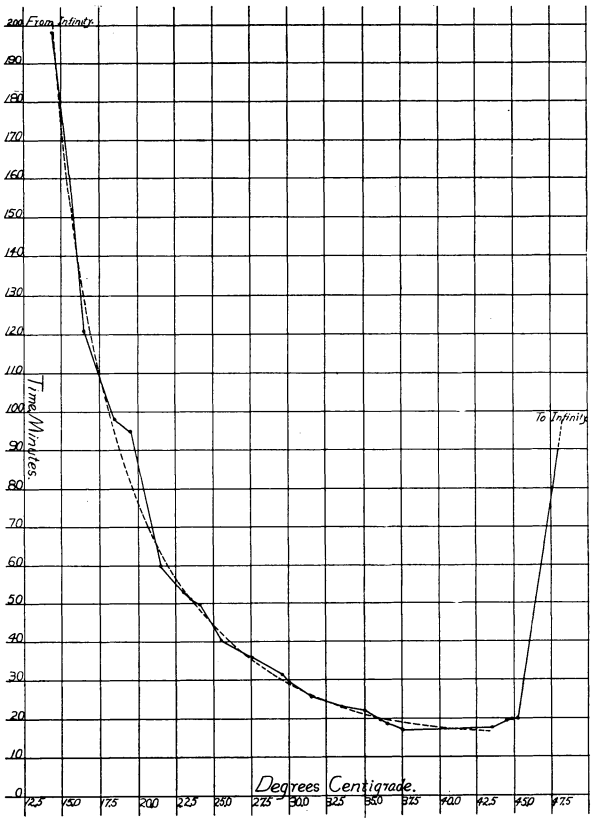

CHART 1.—The growth-rate of *B. coli* at different temperatures. The broken line approximates a curve plotted along the center of gravity of the points determined.

of the first table marked with the asterisk, but some values are taken from other tables also. In the ordinates and abscissae are plotted the time and the temperature respectively. The portion of the curve above 45° and below 12°5 is omitted since the determinations at these temperatures were irregular and uncertain.

The broken line on the chart approximates a curve plotted along

the center of gravity of the points determined. This line does not, apparently, correspond to any simple curve, but the portion extending as far as 45° approximates the quarter of an ellipse. The long diameter of this ellipse, owing to difficulties in obtaining exact data at lower temperatures, is almost impossible to determine, so no attempt is made to definitely determine its nature. The graphical presentation in the chart represents the data obtained probably better than an attempted formulation of an equation for the curve or any part of it, and further mathematical considerations will be deferred until further data on this and other species are obtained.

It is observed that the generation time, beginning at infinity, diminishes rapidly at first, then more gradually, and finally reaches a minimum at about 37° C. From that point there is but little change until about 46° is reached when the rate increases rapidly to reach infinity at 50°.

The results shown in this curve tend to confirm the conclusion of Müller[1] that no fever temperature likely to occur in the human body can be high enough to directly inhibit the growth of pathogenic bacteria.

It may be asked how *B. coli* came to be adapted to a relatively rapid growth at temperatures far above body heat and above the temperatures occurring during the warmest summer weather. The explanation may possibly be that this species had adapted itself to growth in the higher temperatures occurring in fermenting masses of organic material. It is certain that in this species there is only a narrow margin between the point at which the maximum rate of reproduction ceases and the thermal death point of a large proportion of the individuals. Even at 45° growth was less regular, and there is an increase in the proportion of bacteria which fail to grow. It is possible that bacilli might be isolated which, under the most favorable conditions, would show at temperatures above 45° the rapid rate characteristic of temperatures below 45° and that the maximum rate of reproduction is closer to the thermal death point than is indicated in my experiments.

These experiments indicate also that in presumptive tests for *B. coli* in drinking water, the incubation temperature should not exceed

[1] *Ztschr. f. Hyg.*, 1895, 20, p. 245.

45° C. The following experiments were carried out to test these temperatures. Water, polluted by sewage, was plated in lactose litmus agar and incubated at 46°.3, 47°.3, and at 48°.3. Controls were incubated at 37°. The controls showed by far the larger numbers of presumptive coli colonies in all three experiments.

From the lowest temperatures tested to about 40° the bacilli were for the most part actively motile. In some hanging drops and in test-tube experiments motility apparently continued actively when the temperature was too low for reproduction. Above 40° the large majority of the bacilli were non-motile, though the rate of reproduction was as rapid as with the motile bacilli at lower temperatures. Occasionally, at temperatures below 40 degrees motility would be absent during the whole time of an experiment, with no apparent diminution of the growth rate. Apparently there is little or no correlation between motility and the rate of reproduction in this species.

The morphology also varied somewhat with the temperature. In freshly growing cultures at temperatures below 40° there was a tendency to break up into relatively long units. In the higher temperatures, especially 45°, there was a tendency to irregularity. Some individuals tended to form long filaments while others, in the same drop, it might be, broke up into very small elements. The short elements tended at these higher temperatures to break up into new elements like themselves, thus resembling the units formed in older cultures at lower temperatures.

The media used in these experiments have been various sorts of beef broth made from meat infusions. The most of the experiments were made with the ordinary beef peptone broth, in some cases plain, and in others modified by the addition of 1 per cent of glucose. It was found that the addition of the sugar made very little difference. The omission of salt made no appreciable diminution in the rate. Infusions of finely chopped lean meat were used in all cases. Broth from meat taken immediately after the slaughter of the animal gave no better results than that made from meat for some time preserved in cold storage. All broths used were acid to phenolphthalein, but various degrees of acidity were tested. The best results were obtained with a broth about 1 per cent acid. Considerable acid, even to 2 per

cent, made but little difference in the rate. Various combinations of beef broth with sterile unheated human serum, with the juices of liver unheated, and extract of muscle unheated, gave no more rapid rate. There was apparently a less rapid rate in fresh human serum in broth than when this serum had stood in the broth for some days. No attempt was made to carefully compare the values of different media further than to ascertain what media gave the optimum conditions. It is quite possible that a medium may be found which would give a still lower generation times than those which I have determined.

All of the experiments described above were made with *B. coli.* It is recognized that another species, or even another race of *B. coli*, might give a very different temperature curve. Little was done with other species of bacteria in my experiments except a few determinations on *B. typhosus* and on *B. subtilis.* The last species was chosen to ascertain if the reproduction rate of a comparatively large species equals that of smaller species. In the warm box and in the hanging drops *B. subtilis* gave a generation time about 20 minutes at nearly 37°. One race of typhoid at 36°.6 gave in the warm box 27 generations in 720 minutes, a generation time of 26.7 minutes.

In comparing the results obtained in the above experiments with those of other writers we find that our results, as a rule, show a more rapid rate of reproduction and more uniform values of *G.* Buchner obtained for Asiatic cholera grown at 37°.7 results ranging from $G=19.7$ to $G=40.0$ min. Rahn obtained for *B. coli* a minimum of $G=37$ minutes at 27°. For *B. fluorescens liquefaciens* he obtained $G=60$ minutes at 25° and 30.0 minutes at 28°. Hehewerth obtained for *B. coli* at 37° an average value of 23 minutes 24 seconds with a minimum two minutes or so lower. At 22° he obtained for *B. coli* 77 minutes 54 seconds. For *B. typhosus* at 37° he obtained an average value of 33 minutes 24 seconds. Müller obtained for *B. typhosus* at the temperature 37°.5–38°.1 an average of 32.02 minutes and a minimum of 28.65 minutes. For his bacillus A he obtained the following series:

30 degrees	46 min.	38 sec.
25 degrees	51 min.	24 sec.
6 degrees	7 hrs.	36 min.
0 degrees	19 hrs.	57 min.

For his bacilli B, C, and D, and *B. fluorescens liquefaciens* he obtained somewhat similar ratios.

SUMMARY.

The reproduction of *Bacillus coli*, beginning at about 10°, increases rapidly to about 37° C. where it reaches its maximum rate with a generation time of nearly 17 minutes. This rate is nearly constant until the temperature of 45° when it falls rapidly and reproduction practically ceases at 49°.

The initial slow growth described by various authors as occurring when small numbers are transferred to fresh sterile medium does not occur in this species, if an actively dividing bacillus accustomed to the medium is used as a starter.

Under constant conditions the rate of growth remains constant at least as far as the thirty-eighth generation, and all offspring continue to divide at the same geometrical ratio as the parent bacilli.

It is not probable that any body temperature during fever can be high enough of itself to materially alter the rate of reproduction of pathogenic bacteria.

There is with *B. coli* little or no correlation between motility and rapidity of division.